Uptake and degradation of human low-density lipoprotein by human liver parenchymal and Kupffer cells in culture

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The association with and degradation by cultured human parenchymal liver cells and human Kupffer cells of human low-density lipoprotein (LDL) was investigated in order to define, for the human situation, the relative abilities of the various liver cell types to interact with LDL. With both human parenchymal liver cells and Kupffer cells the association of LDL with the cells followed saturation kinetics which were coupled to LDL degradation. The association of LDL (per mg of cell protein) to both cell types was comparable, but the association with human Kupffer cells was much more efficiently coupled to degradation than was the case in parenchymal cells. The capacity of human Kupffer cells to degrade LDL was consequently 18-fold higher (per mg of cell protein) than that of the human parenchymal liver cells. Competition studies showed that unlabelled LDL competed efficiently with the cell association and degradation of ^{125}I -labelled LDL with both parenchymal and Kupffer cells, while unlabelled acetyl-LDL was ineffective. The degradation of LDL by parenchymal and Kupffer cells was blocked by chloroquine and NH_4Cl , indicating that it occurs in the lysosomes. Binding and degradation of LDL by human liver parenchymal cells and human Kupffer cells appeared to be completely calcium-dependent. It is concluded that the association and degradation of LDL by human Kupffer and parenchymal liver cells proceeds through the specific LDL receptor, whereas the association of LDL to Kupffer cells is more efficiently coupled to degradation. The presence of the highly active LDL receptor on human Kupffer cells might contribute significantly to LDL catabolism by human liver, especially under conditions whereby the LDL receptor on parenchymal cells is down-regulated.

INTRODUCTION

Low-density lipoprotein (LDL) is the major carrier of cholesterol in man. The major part of LDL is cleared from the plasma via the LDL-receptor-mediated pathway. The liver plays a key role in the process of receptor-mediated endocytosis of LDL because it is the major site of expression of LDL receptors and it is the only organ in which cholesterol can be removed from the circulation and degraded to form bile acids [1]. LDL receptors have been identified in the livers of a variety of animals, including rats [2], rabbits [3], puppies [4] and swine [5]. In the human, liver LDL receptors have been demonstrated in adult liver membrane preparations [6–8] and in primary cultures of human parenchymal liver cells [9–12]. From the studies with human parenchymal liver cells in culture, it was concluded that the catabolism of LDL proceeds in part through an LDL-receptor-mediated pathway similar to that demonstrated on extrahepatic cells.

Studies performed with rats [13,14] and rabbits [15] showed that besides parenchymal liver cells, the non-parenchymal liver cells (especially Kupffer cells) play an important role in the uptake of LDL. In rats, the non-parenchymal cells account for 71% of the total liver uptake of LDL, and within the non-parenchymal cell population the Kupffer cells are responsible for the receptor-dependent uptake of LDL [14,16]. In rabbits, non-parenchymal cells, depending on the concentration of circulating LDL, are responsible for 8–26% of the hepatic uptake of LDL [15]. Although studies have been performed on the isolation and culture of human Kupffer cells [17], no information is available on the ability of human Kupffer cells to interact with LDL. In order to characterize the interaction of LDL with human liver cells and to determine the possible relevance of human Kupffer cells in the metabolism of LDL, we performed studies on the

interaction of LDL with human liver parenchymal cells and human Kupffer cells in culture.

MATERIALS AND METHODS

Materials

Collagenase (type I), BSA (fraction V), dexamethasone and chloroquine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ^{125}I was purchased from Amersham International (Amersham, Bucks., U.K.), and Nycodenz was from Nycomed A/S (Oslo, Norway). Fetal calf serum, penicillin and streptomycin were obtained from Boehringer (Mannheim, Germany). Williams' E culture medium and kanamycin were from Flow Laboratories (Irvine, Scotland, U.K.), RPMI 1640 cell culture medium was from Gibco (Paisley, Scotland, U.K.) and multi-well cell culture dishes were from Costar (Cambridge, MA, U.S.A.). All other chemicals were of analytical grade.

Isolation, modification and labelling of LDL

Human LDL was obtained from the blood of healthy volunteers who had fasted overnight. Isolation of LDL ($1.024 < d < 1.055$) was performed according to Redgrave *et al.* [18]. LDL was acetylated by repeated additions of acetic anhydride as described before [19]. LDL was radiolabelled with ^{125}I by the ICI method of McFarlane [20], as modified by Bilheimer *et al.* [21].

Isolation of human liver cells

Human liver cells were isolated from livers which were obtained through the Auxiliary Liver Transplantation Program, which is carried out at the University Hospital Dijkzigt in Rotterdam,

Abbreviation used: LDL, low-density lipoprotein.

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Fig. 1. Relationship between the concentration of ^{125}I -LDL in the absence or presence of unlabelled LDL (300 $\mu\text{g/ml}$) and the extent of association and degradation of LDL by human parenchymal and Kupffer cells

Cells were incubated for 3 h at 37 °C with the indicated amounts of ^{125}I -LDL in the presence (broken line) or absence (●) of 300 μg of unlabelled LDL/ml. Receptor-specific association and degradation (■) was derived by subtracting non-specific association or degradation from total association or degradation. Association (a,c) and degradation (b,d) of ^{125}I -LDL by parenchymal cells (a,b) and Kupffer cells (c,d) are expressed as ng of apolipoprotein associated or degraded/mg of cell protein.

The Netherlands. Permission was given by the Medical Ethical Committee to use the remaining, not transplanted, part of donor livers for scientific research. The livers were obtained from physically healthy organ donors who died after brain haemorrhages or severe traumatic brain injury. During resection of the left lobe, the liver was perfused by portal vein cannulation with Euro Collins (4 °C).

After resection, the left liver lobe was transported to the perfusion site within 45 min in cold buffer (4 °C) containing 10 mM-Hepes, 142 mM-NaCl, 16.7 mM-KCl and 0.5 mM-EGTA (pH 7.4). Perfusion at 37 °C with 3 litres of this buffer, which was oxygenated, at a rate of 40 ml/min per catheter was started after insertion of between one and four polyethylene catheters (18 gauge) in the vascular orifices, which could be identified at the dissection surface. After the pre-perfusion, the liver was perfused successively (1) with 500 ml of a Hepes buffer (pH 7.6) containing 5 mM- CaCl_2 without recirculation, (2) with 200 ml of this buffer containing 0.05% collagenase and (3) with 200 ml of this buffer containing 0.1% collagenase (2 and 3 with recirculation), for 20 min each. Liver tissue was dissociated in a Hanks buffer containing 0.2% BSA; cells were filtered through a 250 μm -pore-size filter, centrifuged (50 g for 30 s) and washed three times with cold Williams' E culture medium (4 °C) to remove parenchymal cell debris and non-parenchymal cells [22]. The supernatants of the first centrifugation and the first wash were used for the isolation of non-parenchymal cells. Non-parenchymal cells were isolated by centrifugal elutriation, essentially as described before [23]. Successive fractions were collected at flow rates of 12.5, 25, 39 and 70 ml/min at a rotor speed of 1500 g. The fraction

eluting at 70 ml/min was used for culturing Kupffer cells. Viabilities were examined by a Trypan Blue exclusion test. The purity of cultured cells was examined by electron microscopy. The cells were fixed *in situ* in 2% glutaraldehyde in 0.15 M-cacodylate buffer and further processed for electron microscopy as described earlier [24]. Identification of the cells was based on their morphological and ultrastructural appearance [17].

Culture of human liver cells

Human parenchymal cells were plated in 12-well cluster plates at a density of 0.5×10^6 cells/well in Williams' E medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM-L-glutamine, 20 munits of insulin/ml, 1 nM-dexamethasone, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 50 μg of kanamycin/ml at 37 °C in a humidified CO_2 /air (1:19) atmosphere. Human Kupffer cells were plated in 24-well cluster plates at a density of 1×10^6 cells/well in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal calf serum, 2 mM-L-glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml at 37 °C in a humidified CO_2 /air (1:19) atmosphere. Media were renewed 12 h after seeding to remove non-viable and unattached cells, and every 24 h thereafter. Experiments with cultured parenchymal and Kupffer cells were performed between the 2nd and 6th day after seeding of the cells.

LDL association with and degradation by human liver cells

At 24 h before the start of the experiment, the culture medium was removed and replaced by Williams' E medium containing 1% human serum albumin. Experiments were started by addition

Fig. 2. Comparison of the ability of unlabelled lipoproteins to compete with the cell association and degradation of ^{125}I -LDL in human parenchymal and Kupffer cells

Cells were incubated for 3 h at 37 °C with 10 µg of ^{125}I -LDL/ml and with the indicated amounts of unlabelled LDL (■) or acetyl-LDL (□). ^{125}I -LDL association and degradation are expressed as a percentage of the radioactivity obtained in the absence of unlabelled lipoprotein. The 100 % values for the association (a) and degradation (b) by parenchymal cells were 135.2 ng of LDL/mg of cell protein and 22.9 ng of LDL/mg of cell protein respectively. For Kupffer cells the 100 % value for association (c) was 188.1 ng of LDL/mg of cell protein and for degradation (d) it was 751.9 ng of LDL/mg of cell protein.

of culture medium containing the indicated amounts of ^{125}I -LDL, 1 % human serum albumin and indicated additions. Cells were incubated for 3 h at 4 °C to determine ^{125}I -LDL binding or at 37 °C to determine association and degradation as indicated. After incubation, culture plates were placed on ice and 0.5 ml of the medium was taken to measure degradation of ^{125}I -LDL as described before [25]. Cells were washed five times with ice-cold buffer containing 0.15 M-NaCl, 50 mM-Tris/HCl, 2.5 mM- CaCl_2 (pH 7.4) and 0.2 % BSA, followed by two washes with the same buffer without BSA. The cells were then dissolved in 0.1 M-NaOH and cell-associated radioactivity was determined. Protein content was measured according to Lowry *et al.* [26].

RESULTS

Culture of human parenchymal and Kupffer cells

The yield of parenchymal cells varied per isolation from 640×10^6 to 4790×10^6 cells. This wide range can be explained by the variation in size and shape of the pieces of human liver that were used for cell isolation. The viability of the parenchymal cells directly after isolation was 70.2 ± 10.8 % ($n = 5$). The viability of the cultured parenchymal cells used in this study was higher than 95 %, because non-viable cells do not attach to the culture dishes. During the first 2 days in culture, isolated parenchymal cells

Fig. 3. Effects of chloroquine and NH_4Cl on the association and degradation of ^{125}I -LDL by human parenchymal and Kupffer cells

Cells were incubated for 3 h at 37 °C with 10 µg of ^{125}I -LDL/ml in the presence of 50 µM-chloroquine (B), 100 µM-chloroquine (C) or 10 mM- NH_4Cl (D), or without additives (A). ^{125}I -LDL association and degradation are expressed as percentages of the radioactivity obtained in the absence of chloroquine or NH_4Cl . The 100 % values for association (a) and degradation (b) by parenchymal cells were 135.2 ng of LDL/mg of cell protein and 22.9 ng of LDL/mg of cell protein respectively. For Kupffer cells the 100 % value for association (c) was 178.2 ng of LDL/mg of cell protein and for degradation (d) it was 762.8 ng of LDL/mg of cell protein.

formed a monolayer with a morphological appearance characteristic of liver parenchymal cells (monitored by phase-contrast microscopy). Cells maintained these characteristics for at least 9 days. The purity of the cultured parenchymal cells was higher than 99 %, as examined by electron microscopy. The yield of the Kupffer cell fraction which was purified from the non-parenchymal cell mixture by centrifugal elutriation varied from 50×10^6 to 310×10^6 cells, with a viability higher than 95 % directly after isolation. Upon plating, Kupffer cells attached very readily to the dishes and the maintenance culture could be retained for at least 7 days. The purity of Kupffer cells in culture, as examined by electron microscopy, was above 70 %. Other cell types present in the cultures were mostly white blood cells (8–26 %) and occasionally parenchymal cells (0–6 %), liver endothelial cells (0–6 %) or fat-storing cells (0–1 %).

Association and degradation of ^{125}I -LDL by human parenchymal and Kupffer cells

The amount of cell association as a function of extracellular ^{125}I -LDL concentration in the presence or absence of excess unlabelled LDL is shown in Fig. 1. These data show, for both parenchymal and Kupffer cells, that the association of ^{125}I -LDL with these cell types is saturable. This is especially evident when the interaction in the presence of excess unlabelled LDL (300 µg/ml) is subtracted from the total cell association. The high-affinity cell association to parenchymal cells at 3 h of incubation was 400 ng/mg of cell protein, while for Kupffer cells a value of 482 ng/mg of cell protein was obtained. The high-affinity association of LDL to parenchymal and Kupffer cells is coupled to the degradation of LDL. With parenchymal cells, a

Fig. 4. Effects of Mg-EGTA and Ca²⁺ on cell binding and degradation of ¹²⁵I-LDL by human parenchymal (a,c) and Kupffer cells (b,d)

Cells were incubated for 3 h at 4 °C (binding; a,b) or at 37 °C (degradation; c,d) with 10 µg of ¹²⁵I-LDL/ml and with the indicated Mg-EGTA or free Ca²⁺ concentrations. Binding and degradation are expressed as percentages of the binding, association or degradation at 1.8 mM-Ca²⁺.

maximal value for high-affinity degradation of 62 ng/mg of cell protein was achieved, whereas for Kupffer cells this value was 1107 ng/mg of cell protein. Thus it appears that Kupffer cells in culture possess a high-affinity capacity (per mg of cell protein) to degrade LDL which is 18-fold higher than that for parenchymal cells.

With increasing incubation time up to 2 h, the association of ¹²⁵I-LDL to parenchymal cells proceeded linearly, followed by a slight further increase between 2 and 3 h of incubation. The degradation of ¹²⁵I-LDL by human parenchymal cells showed a clear lag phase of 30 min, and then degradation of ¹²⁵I-LDL proceeded linearly between 1 and 3 h. The time-dependency of both the association and the degradation of ¹²⁵I-LDL by Kupffer cells followed a similar pattern. A comparison of the cell association at 37 °C and 4 °C (binding) of parenchymal and Kupffer cells reveals information about the relationship between binding and cell uptake in the two cell types. Association of ¹²⁵I-LDL (10 µg/ml) with parenchymal cells at 37 °C was 188.1 ± 41.6 ng of LDL/mg of cell protein and at 4 °C it was 39.4 ± 12.8 ng of LDL/mg (means ± S.D., *n* = 9). The association with Kupffer cells at 37 °C and 4 °C respectively was 173.1 ± 17.8 and 31.9 ± 6.1 (*n* = 5) ng of LDL/mg of cell protein. These data indicate that the cell association at 37 °C is 4.8-fold (parenchymal cells) or 5.4-fold (Kupffer cells) higher than the binding determined at 4 °C.

The specificity of the interaction of native LDL with human parenchymal and Kupffer cells was determined by incubating these cell types with ¹²⁵I-LDL and increasing amounts of unlabelled native LDL or acetyl-LDL (Fig. 2). It is clear that native LDL is an effective inhibitor of both the cell association and the degradation of ¹²⁵I-LDL by parenchymal cells as well as by Kupffer cells. In contrast, acetylation of LDL leads to a loss of competitive ability, as acetyl-LDL appears to be ineffective.

Effects of chloroquine and NH₄Cl on the processing of ¹²⁵I-LDL by human parenchymal and Kupffer cells

The effects of chloroquine and NH₄Cl on the association and degradation of ¹²⁵I-LDL by parenchymal and Kupffer cells are shown in Fig. 3. Degradation of ¹²⁵I-LDL by parenchymal cells was inhibited by 80 % by 50 µM-chloroquine, and the presence of 100 µM-chloroquine led to complete inhibition. Also, NH₄Cl was a very effective inhibitor. No change in the cell association of ¹²⁵I-LDL was observed in the presence of these lysosomotropic agents. Degradation of ¹²⁵I-LDL by Kupffer cells was inhibited by 78 % and 86 % by 50 µM- and 100 µM-chloroquine respectively, whereas, with NH₄Cl, 86 % inhibition was recorded. The association of ¹²⁵I-LDL with Kupffer cells was increased to 163 % and 151 % by 50 µM- and 100 µM-chloroquine respectively; NH₄Cl caused a slight decrease in the association of ¹²⁵I-LDL with Kupffer cells.

Effects of Mg-EGTA and Ca²⁺ on the binding and degradation of ¹²⁵I-LDL by human parenchymal and Kupffer cells

The effects of Mg-EGTA and Ca²⁺ upon cell binding and degradation of LDL by parenchymal cells and Kupffer cells are shown in Fig. 4. In both cell types, binding of LDL is largely Ca²⁺-dependent. The degradation of ¹²⁵I-LDL was completely blocked by the presence of 2 mM- or 5 mM-Mg-EGTA. Maximal degradation was observed at low Ca²⁺ concentrations, while with both parenchymal and Kupffer cells at higher Ca²⁺ concentrations sub-optimal degradation of LDL occurred.

DISCUSSION

In the present work we compared the interactions of ¹²⁵I-LDL with human parenchymal and with human Kupffer cells in culture. Although some studies on the interaction of LDL with

human parenchymal cells have been performed previously [9–12], so far there are no data on the relative roles of human Kupffer cells and parenchymal cells in LDL metabolism. Because a species difference in the relative importance of Kupffer and parenchymal cells exists for rats [13,14] compared with rabbits [15], it is important to address this question for the human situation. We used livers from physically healthy organ donors, who died after brain injury, to isolate simultaneously parenchymal and Kupffer cells. Both cell types were exposed to collagenase only and Kupffer cells were recovered from the low-speed supernatants of parenchymal cells. The isolation method resulted in cells which readily attached to culture dishes. The purity of the Kupffer cell cultures was comparable with that obtained in a previous described method for human Kupffer cell isolation, in which cells were isolated after a combined Pronase/collagenase perfusion [18]. The contaminating cells were mainly blood cells. It must be realized that perfusion of the human liver occurs under much less favourable conditions than e.g. rat liver, leading to the presence of a larger amount of contaminating cells. Human endothelial liver cells could not be isolated by this method, probably due to the loss of viable endothelial liver cells, which has been observed to occur when cold ischaemic liver material is reperfused with warm oxygenated buffer [27].

The data in this study demonstrate that both human parenchymal cells and human Kupffer cells in culture possess a high-affinity site that recognizes native human ^{125}I -LDL, as shown previously for human parenchymal cells [9–11]. Competition experiments indicate that this LDL recognition site on both cell types is specific for LDL, as no significant competition was observed with chemically modified LDL (acetyl-LDL). Although the amount of association of ^{125}I -LDL with parenchymal and Kupffer cells was comparable, our data show that the degradation of LDL by Kupffer cells per mg of cell protein was 18-fold higher than that by parenchymal cells. This indicates that the association of LDL with Kupffer cells is much more efficiently coupled to catabolism than is the case with parenchymal cells.

A comparison of the amounts of ^{125}I -LDL bound at 4 °C to the parenchymal and Kupffer cells indicates that a difference in the number of binding sites on parenchymal cells versus Kupffer cells cannot explain the higher capacity of the Kupffer cells (per mg of cell protein) to degrade LDL. By using fluorescently labelled LDL, we found recently [28] that human parenchymal cells showed, upon incubation for 3 h at 37 °C, a strong intracellular signal concentration in small vacuolar vesicles. Thus it appears that parenchymal cells readily internalize LDL, but that the fusion of endocytotic vesicles with primary lysosomes and/or the lysosomal degradation of LDL is apparently much less efficient than with Kupffer cells. This difference might be caused by the much higher concentration of lysosomal enzymes in Kupffer cells compared with parenchymal cells, as was shown for rat liver [29,30].

Since the classical LDL-receptor-mediated pathway, as described by Brown & Goldstein [1], involves lysosomal degradation of LDL, we used chloroquine and NH_4Cl to investigate the intracellular processing of ^{125}I -LDL by human liver cells. These unrelated compounds inhibit lysosomal degradation [31]. Both chloroquine and NH_4Cl inhibited the degradation of ^{125}I -LDL by parenchymal and Kupffer cells. Association of ^{125}I -LDL to parenchymal cells was not altered, but association of ^{125}I -LDL to Kupffer cells increased with chloroquine treatment. This could be a result of a combination of the large amount of ^{125}I -LDL that can be handled by human Kupffer cells and the inhibited degradation of ^{125}I -LDL, which may cause intracellular accumulation of LDL. With NH_4Cl treatment of Kupffer cells, the

total amount of LDL handled by these cells was decreased. This might imply that NH_4Cl can inhibit the fusion of endocytotic vesicles with lysosomes in Kupffer cells, as was shown previously for the interaction of lysosomotropic agents with rat parenchymal cells [32].

Ca^{2+} -dependence of the interaction of ^{125}I -LDL with parenchymal cells and Kupffer cells was studied using EGTA to chelate specifically Ca^{2+} in the presence of Mg^{2+} . Mg -EGTA did not cause detachment of human parenchymal cells from their substratum, as has been reported before for EDTA or Ca^{2+} -free medium [10]. We found an inhibition by Mg -EGTA of ^{125}I -LDL binding and degradation by parenchymal and Kupffer cells. This inhibition was overcome by the presence of even a very low concentration of free Ca^{2+} . This is in agreement with previous studies performed with human fibroblasts [33].

In conclusion, our data demonstrate that human parenchymal and Kupffer cells both have the capacity to interact with LDL via a receptor with characteristics similar to those described in fibroblasts [1]. This contrasts with data obtained with rat parenchymal cells, which possess hardly any LDL receptors *in vivo*. Although LDL receptors can be induced in rat parenchymal cells *in vitro* [34], the maximal amount of ^{125}I -LDL binding to the cells is only 40 ng/mg of cell protein, whereas the amount of LDL degraded by rat parenchymal cells was about 1 ng/h per mg of cell protein. Thus it appears that human parenchymal cells more readily interact with LDL than do rat parenchymal cells.

The capacity of human Kupffer cells to degrade LDL is 18-fold higher (per mg of cell protein) than for human parenchymal cells. However, 92.5 % of liver protein consists of parenchymal cells and 2.5 % comprises Kupffer cells [35]. Assuming that human liver endothelial and fat-storing cells do not perform LDL-receptor-mediated uptake (as is shown for rats [14]), it can be calculated that 67 % of the liver's capacity to degrade LDL resides in parenchymal cells. As the LDL receptor of parenchymal cells might be subject to regulation by dietary cholesterol, it is likely that, although the contribution of the LDL receptor from Kupffer cells under optimal culture conditions is only 33 %, Kupffer cells may turn out to be the major site for catabolism of LDL under diet-induced down-regulation of the LDL receptor in the liver. Further experiments, in which the regulation of LDL receptors from human parenchymal and Kupffer cells is compared, should clarify which cell type is mainly responsible for LDL turnover in man under various metabolic conditions.

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